Science AAAS

Supplementary Materials for

Dedifferentiation of Neurons and Astrocytes by Oncogenes Can Induce Gliomas in Mice

Dinorah Friedmann-Morvinski, Eric A. Bushong, Eugene Ke, Yasushi Soda, Tomotoshi Marumoto, Oded Singer, Mark H. Ellisman and Inder M. Verma*

*correspondence to: verma@salk.edu

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Other Suplementary Materials for this manuscript includes the following:

Databases S1 as excel file: MicroarrayVerhaakCorrelation

Materials and Methods

Mice

hGFAP-Cre, Sox2-Cre, Nestin-Cre, SynI-Cre, CamK2a-Cre, Rosa26-LacZ and NOD-SCID mice were all purchased from The Jackson Laboratories. All the colonies were bred as per Jackson Laboratories recommendations and maintained in pathogen-free conditions at The Salk Institute. Mice were monitored daily for signs of illness and euthanized when moribund. All the procedures performed in this study were approved by the IACUC.

Plasmids

All shRNA hairpins were first cloned into an NheI site of the p156RRLsin vector (a third generation lentiviral vector) (*24*). Once validated the shRNA were transferred to pTomo H-RasV12 using the BsrgI and ScaI sites. The U6-shp53 construct was cloned in the HpaI site, 5' from the CMV promoter. The shRNAs used in this study were: shp53 5'-gtacatgtgtaatagctcc – 3', shNF1 5' – gagaaccagttcactctaa – 3'.

In vivo vector injections and orthotopic transplants

Lentivirus (1μl, $1x10^9$ IU, prepared as described previously (*25*)) or cells ($3x10^5$ in 1.5 μl) were stereotaxically injected into mice (transgenic or NOD-SCID, respectively) at the age of 8-16 weeks old under anesthesia (similar results were obtained when 6 months old mice were injected). The following coordinates were used (in mm posterior, lateral and dorsal to the bregma): SVZ (1.5, 2.0, 2.3), HP (2.0, 1.5, 2.3), CTX (1, 1, 0.5 or 1.0; 0, 1, 0.5 or 1.0 and 2.0, 1.5, 0.5) and striatum (0, 1.4, 3.0).

Histology and Immunohistochemistry

All mice were perfused with 0.9% NaCl followed by 4% paraformaldehyde in PBS. The brains were collected, fixed overnight and transferred to 30% sucrose in PBS. For fluorescent staining, we cut 40 μm coronal sections on a sliding microtome and obtained the images by confocal laser-scanning microscopy (Leica TCS SP2 ABS); for high resolution mosaic images sagital sections (70 μm) were cut using a vibrating microtome; all sections were processed for standard immunohistochemical staining as previously described (*26*). For hematoxiylin and eosin (H&E) staining the fixed brains were embedded in paraffin and cut into 6-μM sections. We performed immunohistochemistry on paraffinized sections as previously described (*27*). We obtained the images with a Retiga digital camera on a Nikon optical microscope and analyzed them with OpenLab software (Improvision). All the antibodies used are listed in Supplementary Table 3.

Imaging

Large-scale mosaic volumes were collected on a Nikon RTS 2000 multiphoton microscope equipped with a high-precision automated stage (Applied Precision) (*28*). Imaging was performed with a 40X oil objective (NA 1.30). Each tile of the mosaic consisted of a stack of 11 images, with 3 um z steps. The images were collected with the following set of dichroics and filters: dichroic 1: 500LP; blue filter: 450/80; dichroic 2: 560LP; green filter: 525/50; red filter: 605/70. The mosaics were assembled using custom ImageJ plugins (*28*). The DAPI channel was used for automatic stitching of tiles.

Cell culture

Primary astrocytes and neurons were obtained from 3 or 11 days postnatal pups from GFAP-Cre and SynI-Cre mice, and prepared according to published methods (*29, 30*). Astrocytes were maintained in DMEM containing 10% FBS and neurons were cultured in Neurobasal™-A Medium (Gibco) containing Glutamax™ (Gibco) and B-27 supplement (Gibco). Following transduction of either primary astrocytes or neurons, at the early passages, cells were either cultured in the medium described before or in parallel cultured in NSCs medium containing FGF-2. NSCs media was prepared using the following reagents: DMEM/F-12 (Gibco), $NaHCO_3$, Insulin (Sigma), apo-Transferrin (Sigma), Putrescin (Sigma), Sodium Selenite (Sigma), Progesterone (Sigma), and supplemented with 20ng/ml fibroblast growth factor-2 (PeproTech). Neurospheres were passaged by dissociation of the spheres into single cells using TripLE™ Express (Gibco). Reprogramming was determined by the progressive change in morphology and the appearance of neurosphere like structures, followed by confocal microscopy analysis. Five independently derived cultures of each transgenic mouse were studied in the reprogramming experiments. 005 TICs were maintained in N2-supplemented (Invitrogen) DME-Ham's F12 (Omega Scientific) medium containing 20 ng/ml FGF-2, 20 ng/ml epidermal growth factor (EGF, Promega) and 50 μg/ml heparin (Sigma).

Western blotting

Western blot assays were performed as previously described (*31*) using the antibodies listed in Supplementary Table 4 online.

Flow Cytometry

Cells were stained using the antibodies listed in Supplementary Table 4 and analyzed on a BD LSR II flow cytometer (Becton Dickinson) and CELLQuest software. Brain tumors were dissociated using a Neural Tissue Dissociation Kit (Miltenyi Biotec) and cells were sorted using FACS Vantage SE DiVa (Becton Dickinson) sorter.

Microarray analysis

Array data was median normalized and individual transcript values generated using robust multichip average (RMA) probeset summarization with the Affymetrix power tools package. Duplicate transcripts were averaged to generate one unique gene expression value. Gene expression values were mean subtracted, divided by the standard deviation, and rescaled using the mean absolute deviation (MAD). Genes were filtered using an arbitrary MAD cutoff of >= 0.5, leaving 3377 genes. These rescaled gene estimates were used for downstream single sample gene set analysis (SSGSEA).

Standard SSGSEA has been described previously in Verhaak et al (*23*). Modifications were made to SSGSEA to impose correct gene directionality by ranking genes on actual as opposed to absolute expression and scoring up and down genes separately. Molecular subtype gene sets were derived ClaNC centroids provided in Verhaak (or Phillips (*20*)) and divided into up and down gene sets based on their coordinates. Human genes were matched to mouse genes using official gene symbol, Homologene, and Ensemble orthologous gene information. To obtain a final score for a subtype, the score for down genes was subtracted from the up genes.

For gene sets related to defined cell types, a similar procedure was performed. Gene sets were taken from Lei et al (*32*), which were generated from Cahoy et al study (*16*). For

Cahoy gene sets, genes can only be up. For comparative purposes, scores were divided by the maximum possible score given a similarly sized gene set and gene list. Final assignment of Verhaak molecular subtype classification was based on selection of the largest correlation coefficient of the combined Verhaak and Cahoy signature for each sample compared to the average combined signature for each molecular subclass. Average combined signatures were derived from the unified scaled filtered Verhaak data set of 1741 genes and 202 samples by scoring using the modified SSGSEA procedure. Heat maps were generated using Matrix2png (*33*). GEO entry accession GSE35917.

qRT-PCR analysis

qPCR reactions were done with the 7900HT Fast Real-Time PCR System using the SYBR™ Green PCR Master Mix (Applied Biosystems®). Data are presented after normalization with cyclophilin.. The primers for s100a6 and gfap were described previously (*15*), for plagl2 the 5' primer was TATAGGCACATGGCCACCCACT and the 3' primer was TGACGACGGTATCCCAGCTTT. The rest of the primers were obtained from PrimerBank (*34*) and are listed in Supplementary Table 4.

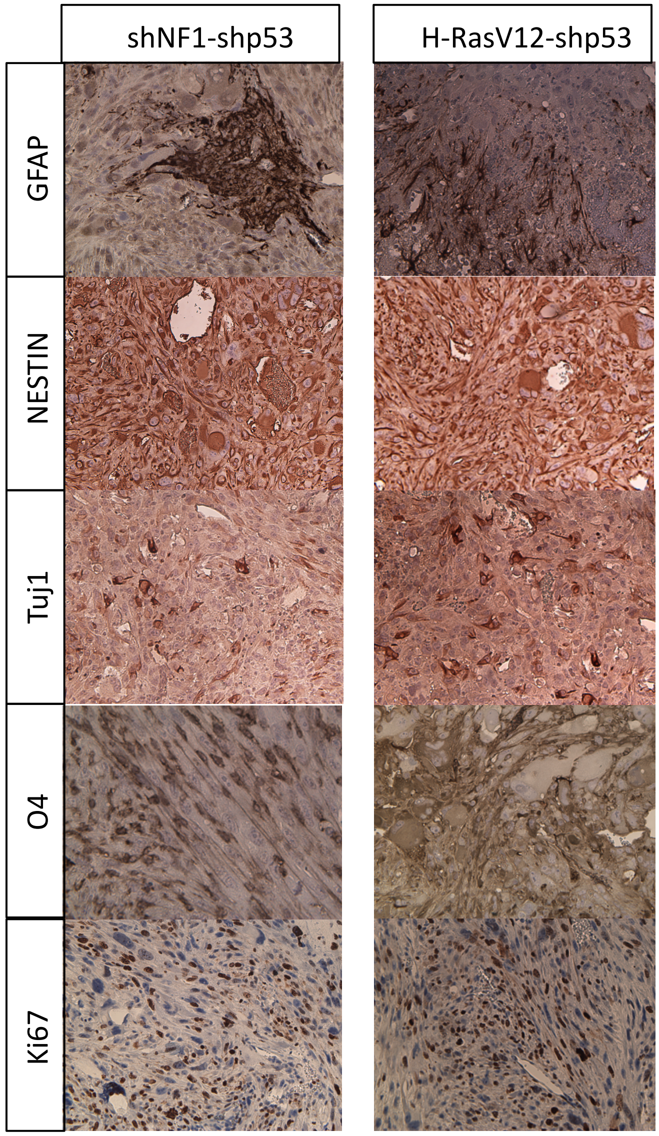


Fig S1. Immunohistochemistry staining of sections from the indicated tumors with antibodies against GFAP, Nestin, Tuj1, O4 and Ki67 (magnification x40).

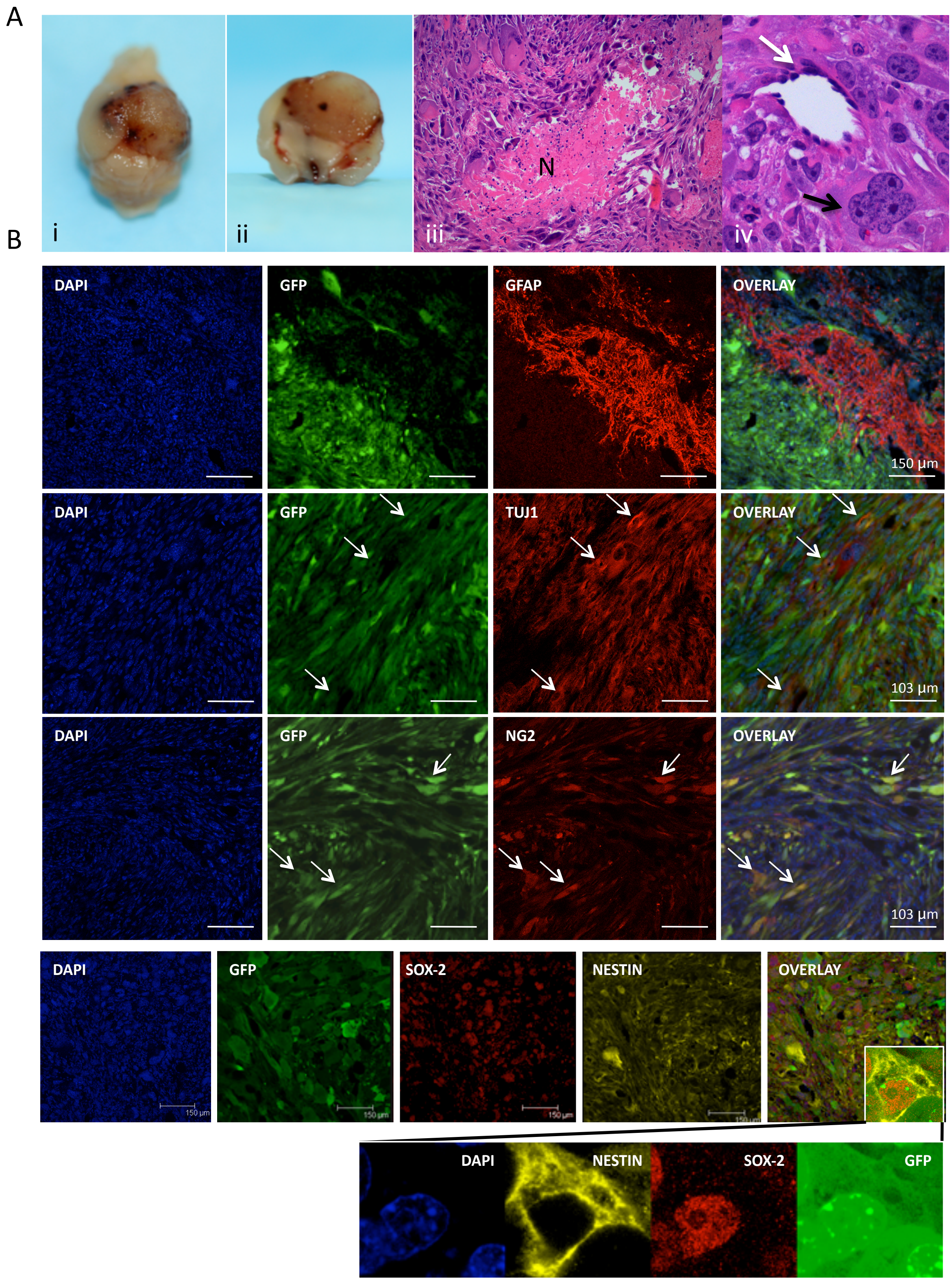


Fig S2. *Gliomas induced by injection of H-RasV12-shp53 in the cortex of SynI-Cre mice.* A. Photographs (panels i-ii) showing the massive lesion in the brain and H&E staining (iii, magnification, x20; iv magnification x40 plus two electrical zoom, showing perivascular infiltration (white arrow) and multinucleated giant cell (black arrow) of the same tumor lesion. B. Tumors sections (40 µm) were analyzed by confocal microscopy using the indicated antibodies, arrows point to representative positive cells. N=necrosis.

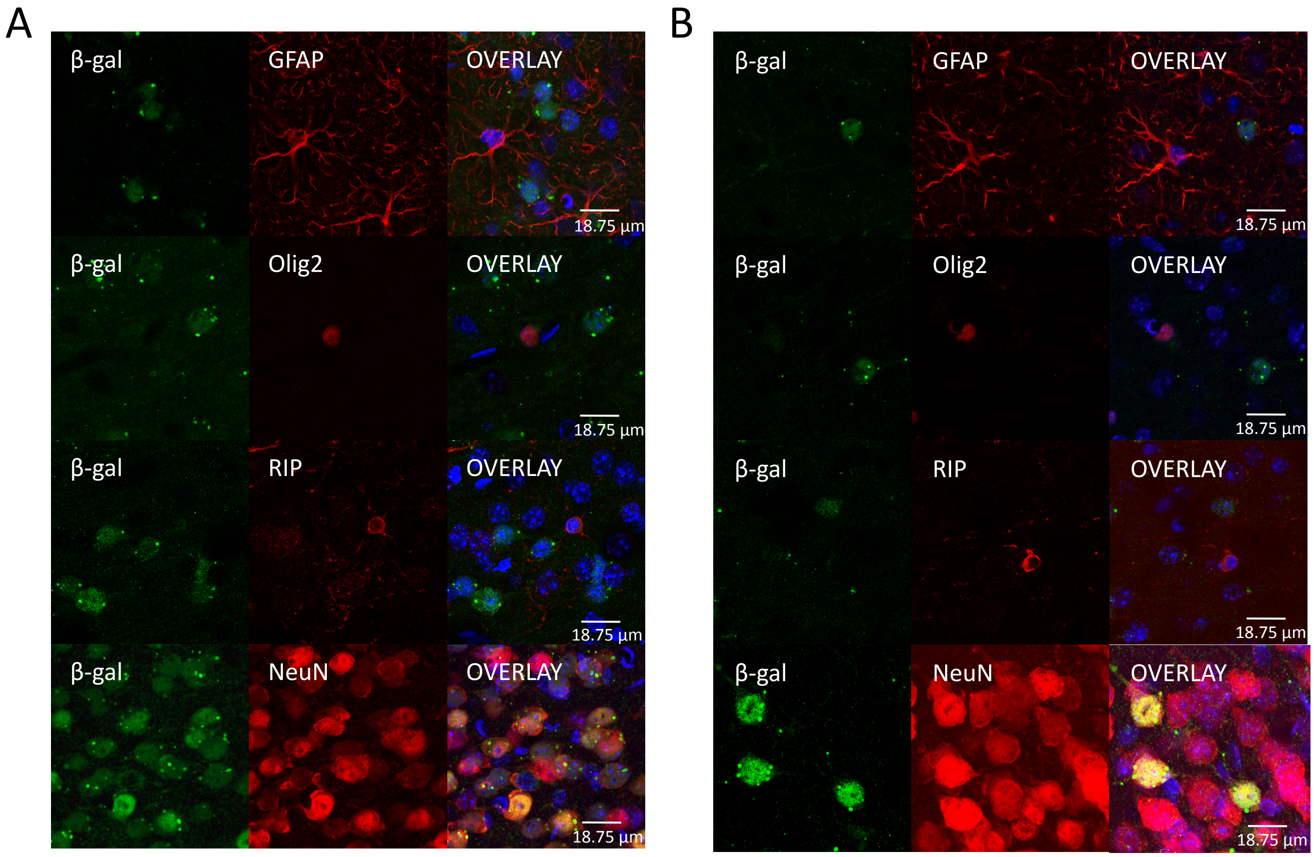


Fig S3. SynI-Cre and *CamK2a-Cre mice express Cre recombinase specifically in neurons*. SynI-Cre (A) and CamK2a-Cre (B) mice were crossed to Rs26-LacZ reporter strain. Double immunofluorsecent analysis with anti-β-gal antibody (green) and the indicated cell marker (GFAP, Olig2, Rip and NeuN; red) shows specific expression of β-gal in NeuN positive neurons in the cortex. Blue = Dapi.

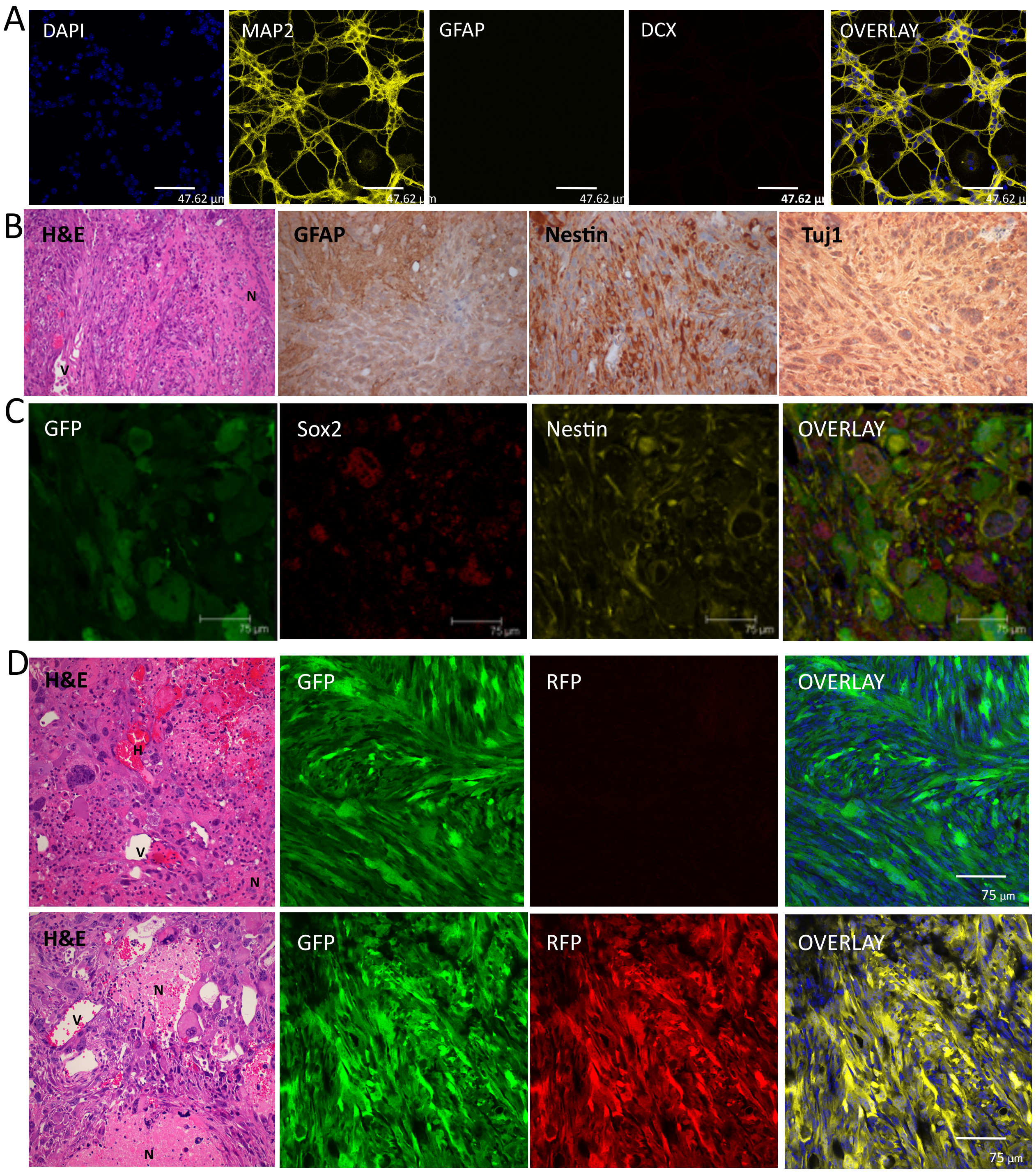


Fig S4. *In vitro transduction of neurons by shNF1-shp53 lentivirus*. A. Confocal analysis of cortical neurons obtained from SynI-Cre mice show positive staining for the neuronal marker MAP2 and negative staining for GFAP and neuronal progenitor marker DCX. B. Tumors derived from shNF1-shp53 transduced neurons orthotopically transplanted into the cortex of NOD-SCID mice; H&E histology and immunohistochemistry. C. Confocal microscopy analysis of the same tumors described in F (green=GFP, red=Sox2, yellow=Nestin, scale bar = 75 µm). D. Tumors obtained by lentiviral injections (see Fig 2 B) were dissociated in single cell suspension and sorted into GFP+/RFP- and GFP+/RFP+ cell populations. By limiting dilution analysis GFP+/RFP- (upper panels) and GFP+/RFP- (lower panels) tumor cells were transplanted into new mice. First images on the left show H&E staining (x40) and the rest of the panels show tumor sections (40 µm) analyzed by confocal imaging (blue=DAPI; scale bar = 75 µm).

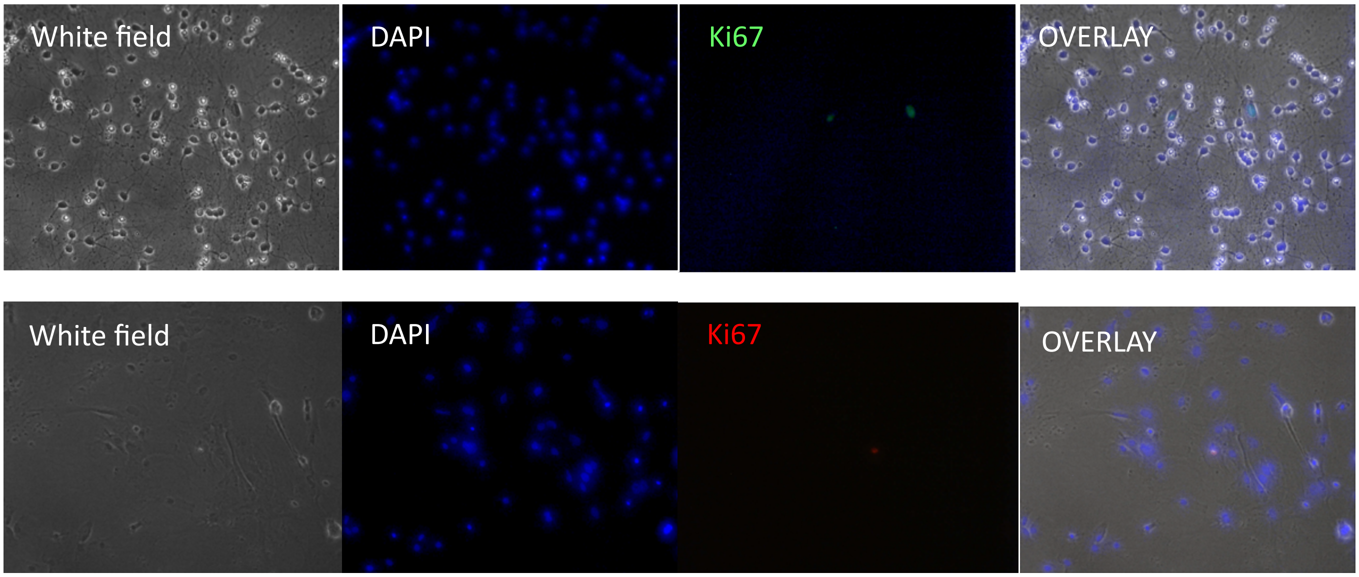
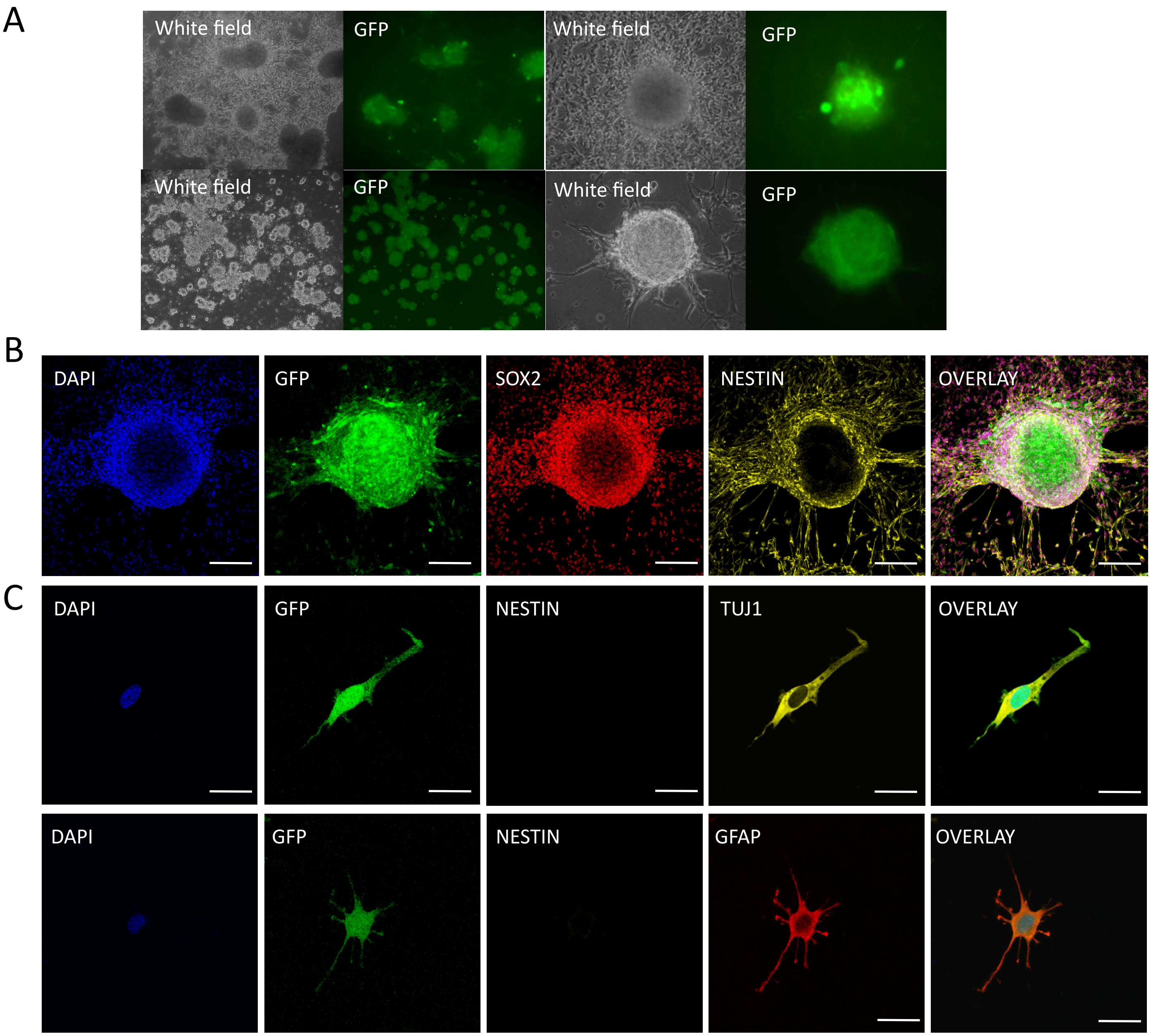


Fig S5. *Neither neurons nor astrocytes isolated from SynI-Cre and GFAP-Cre mice, respectively, stain for Ki67 (proliferation marker)*. Upper panels show neurons in culture (white field) stained with DAPI (blue), and Ki67 (green). Lower panels show astrocytes in culture (white field) stained with DAPI (blue) and Ki67 (red). Magnification x20.



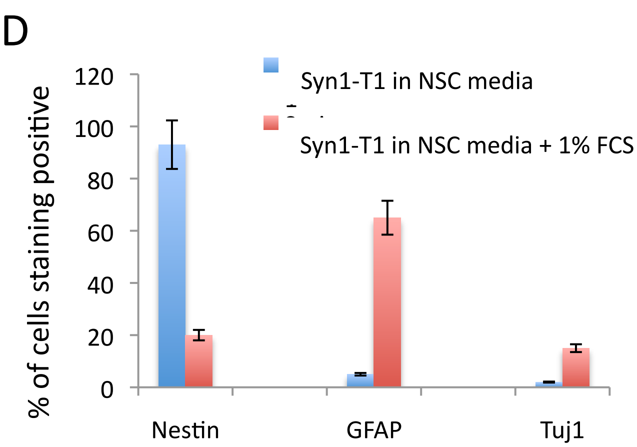
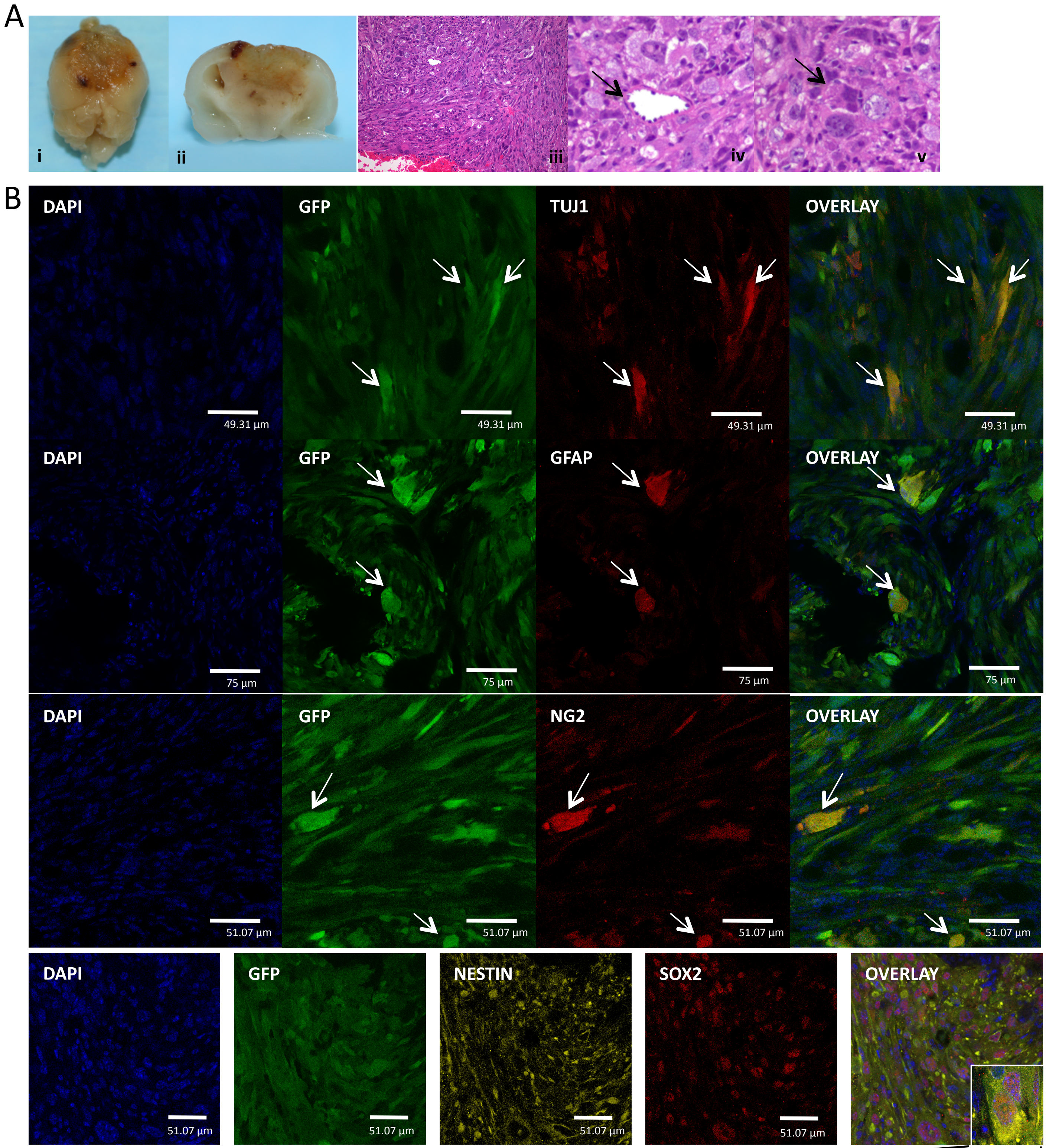


Fig S6. *Syn1-T1 tumor cells show the characteristics of TIC*. A) Neurosphere-like structures formed by Syn1-T1 cells derived from tumors induced by H-RasV12-shp53 in SynI-Cre mice. Upper panels show high (left side, x20) and low (right side, x40) magnification of primary neurospheres and in lower panels secondary neurospheres obtained by single cell/well subculture. B) Confocal microscopy analysis of neurosphere-like structures formed by Syn1-T1; Scale bar = 150µm. C) In response to serum addition in the medium, Syn1-T1 cells express neuronal (Tuj1 +, Nestin -) and astrocytic (GFAP+, Nestin-) markers; Scale bar = 37.5 µm. D) Quantification of the differentiation induced by addition of serum to the media (analyzed 7 days post-addition of serum to the media). Values represent mean ± s.d. from at least three experiments



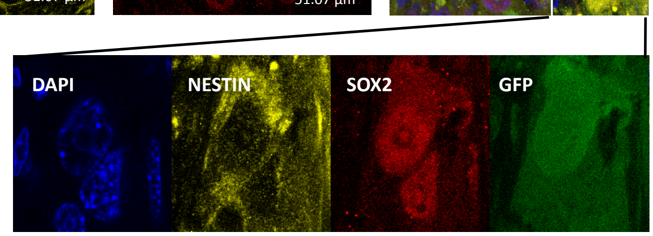


Fig S7. *Gliomas induced by injection of H-RasV12-shp53 in the cortex of CamK2a-Cre mice.* A. Photographs (panels i-ii) and H&E staining (panel iii-v) of tumor lesions. Arrows point to perivascular infiltration in (iv) and multinucleated giant cell in (v). B. Tumors sections (40 µm) were analyzed by confocal microscopy using the indicated antibodies.

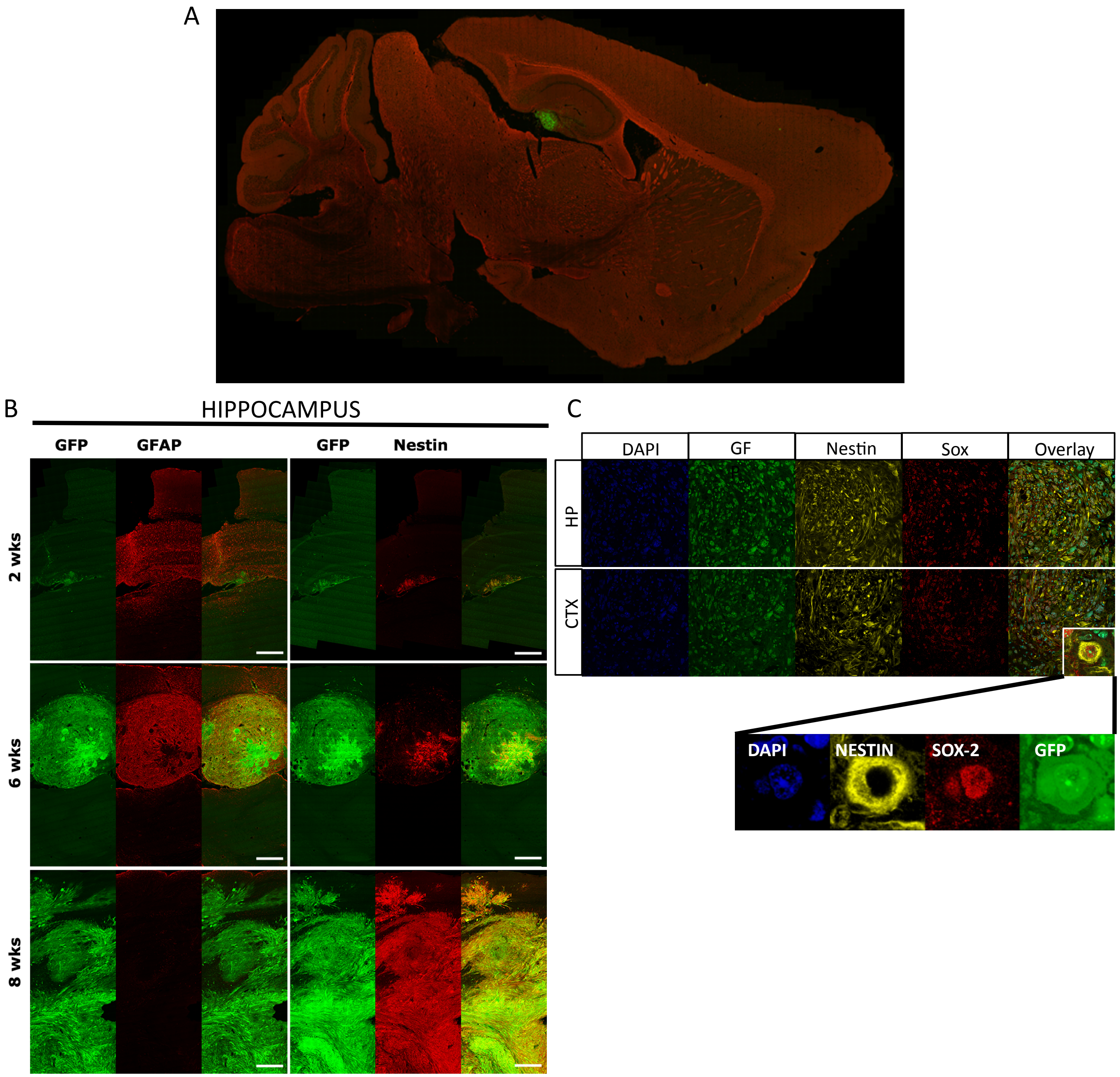


Fig S8. *Tumorigenesis in the hippocampus and CTX of GFAP-Cre mice*. A)Maximum intensity projections of large-scale mosaic volumes. B) Immunolabeling for GFAP and Nestin in tumors at 2, 6, and 8 weeks following injection of lentivirus in the hippocampus. GFAP staining intensity is seen to decrease with tumor progression, while Nestin labeling increases. Scale bars = 500 microns. C) Tumor sections (40 μm) 8 weeks following injection of lentivirus either in the hippocampus (HP) or cortex (CTX) were analyzed by confocal microscopy using Nestin (yellow) and Sox2 (red) antibodies; green=GFP tumor marker, blue=Dapi (magnification x40, inset shows an example of overlay staining in magnification x63 with 2x electrical zoom).

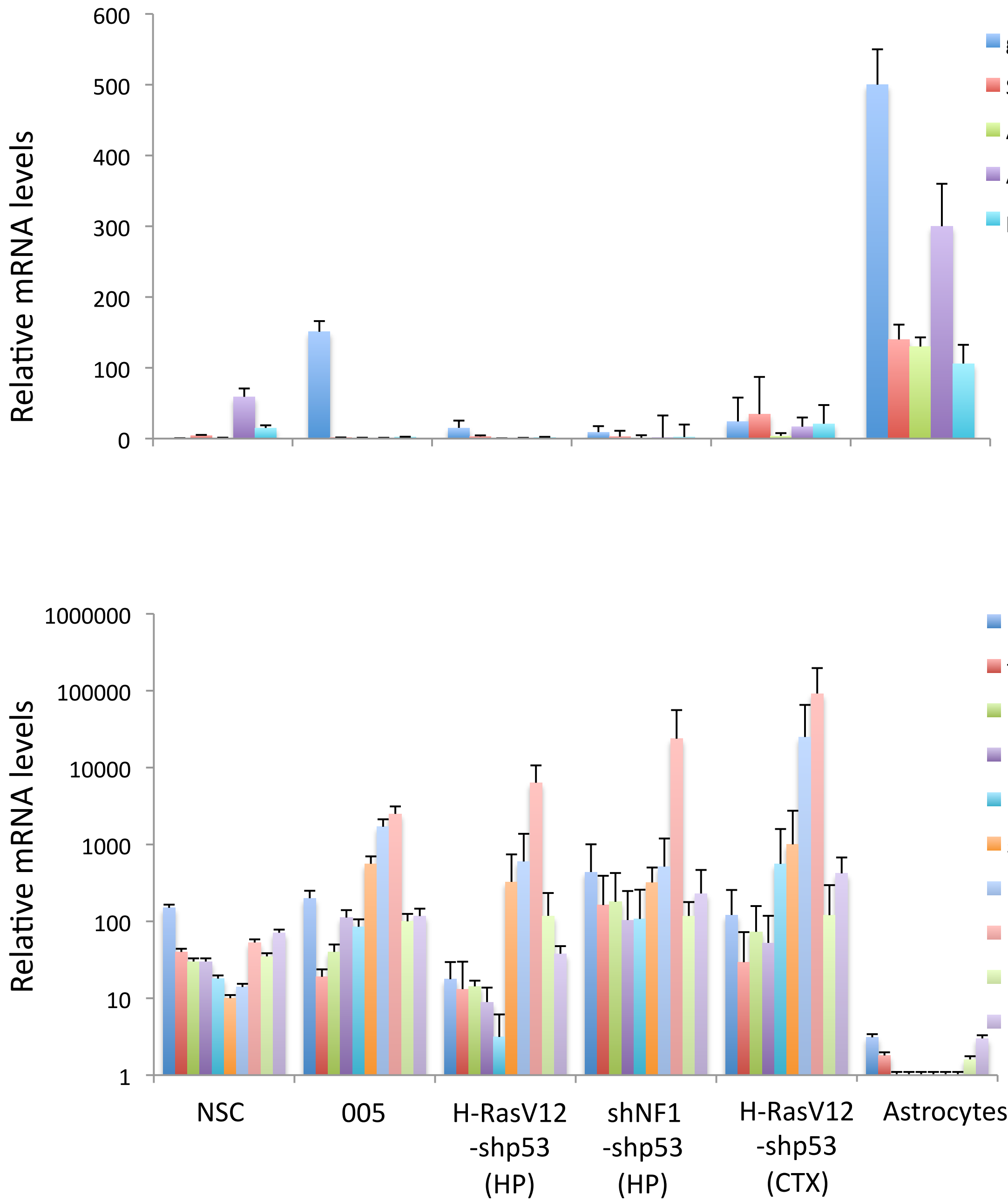


Fig S9. Gene expression analysis by qRT-PCR for ten NSC- and five astrocyte- specific genes in cultured NSC (n=5) , 005 tumor (n=5), H-RasV12-shp53 tumors (n=5, hippocampus (HP) injected) and (n=4, cortex (CTX) injected), shNF1-shp53 tumors (n=4, HP injected) and cultured astrocytes (n=5). Gene expression is normalized to the expression of cyclophilin.

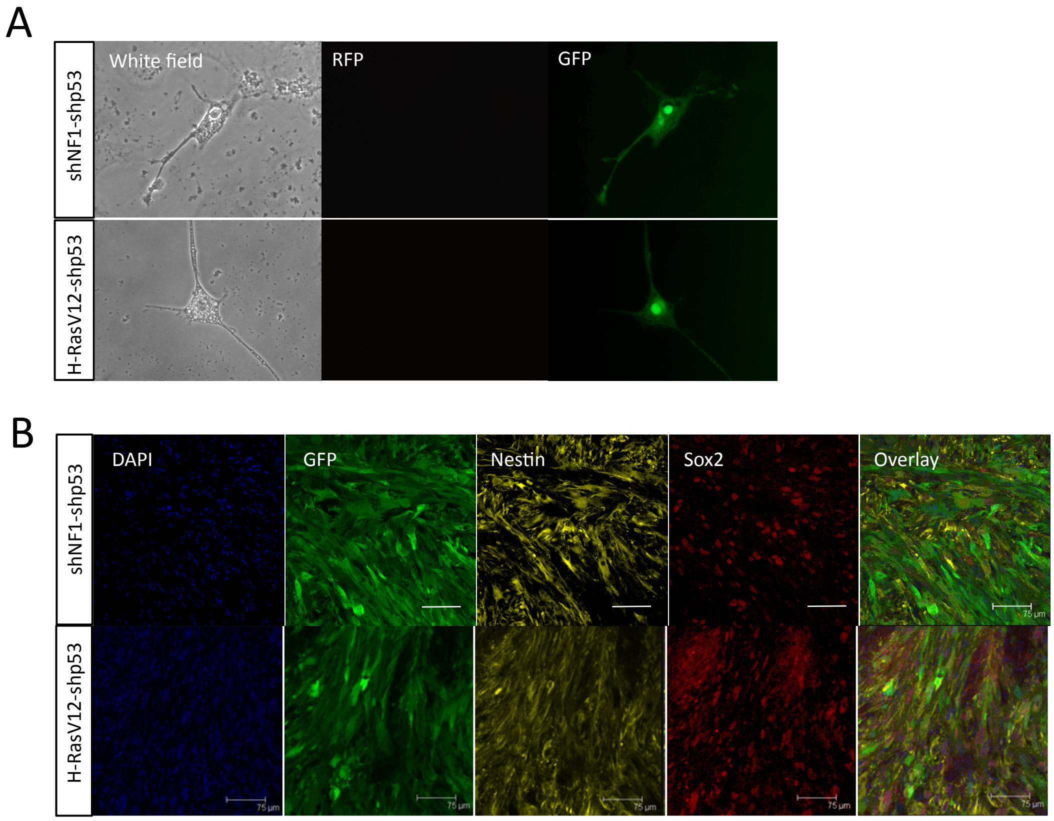


Fig S10. A) Transduced astrocytes express GFP and lost expression of RFP. B) Tumors obtained from transplanting the transduced astrocytes showed high expression of progenitor markers Nestin (yellow) and Sox2 (red) (scale bar = 75 μm).

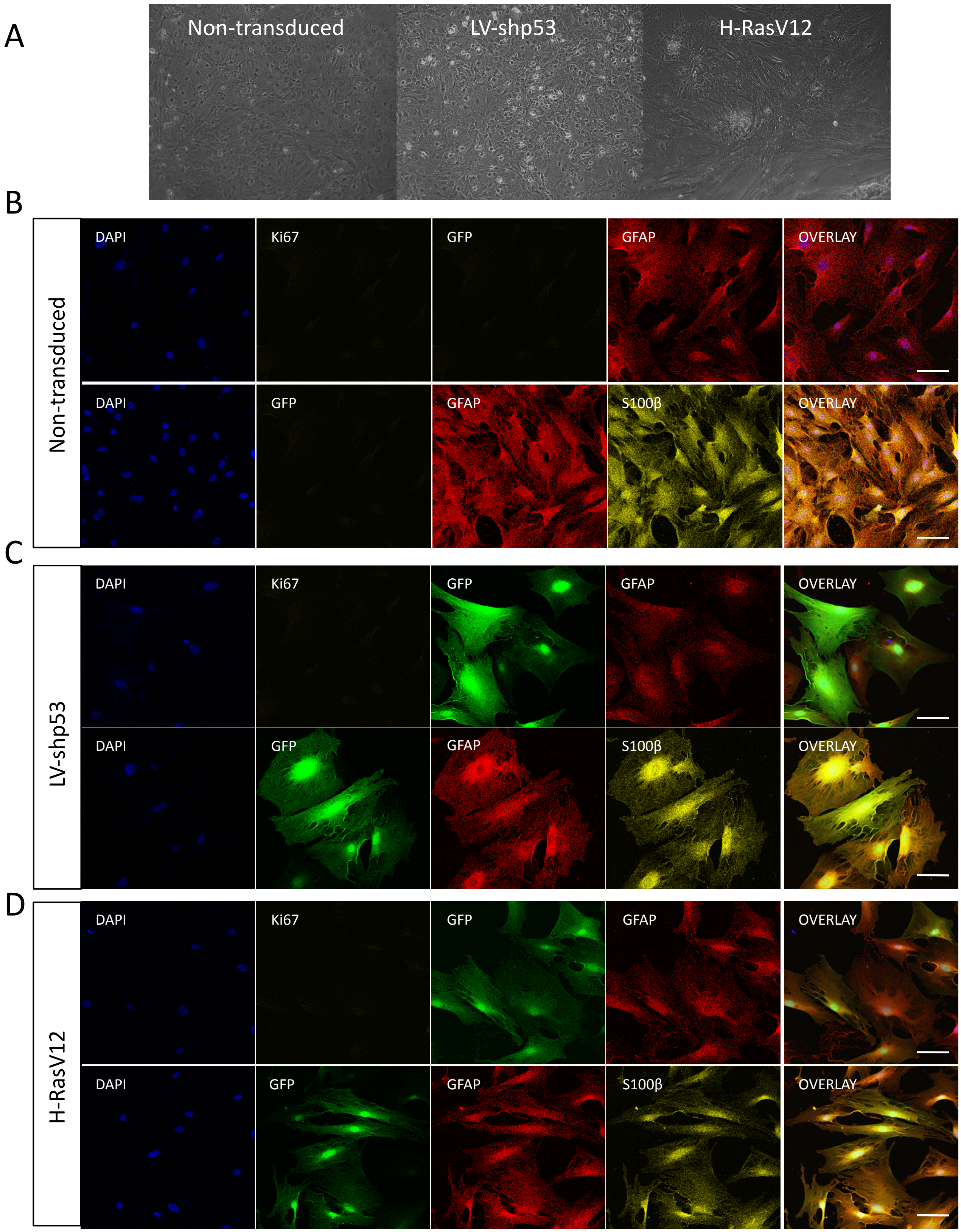


Fig. S11. *White field and fluorescent images of GFAP-Cre derived cortical astrocytes transferred to serum-free media supplemented with FGF-2. A. White field images of astrocytes non-transduced and transduced with the indicated viruses. Magnification x20.* B) non-transduced cells, C) LV-shp53 transduced astrocytes, D) LV-H-RasV12 transduced astrocytes, all stained with the indicated antibodies and analyzed by confocal microscopy. Scale bars = 75 µm. No tumors were obtained when these cells were transplanted to NOD-SCID brains.

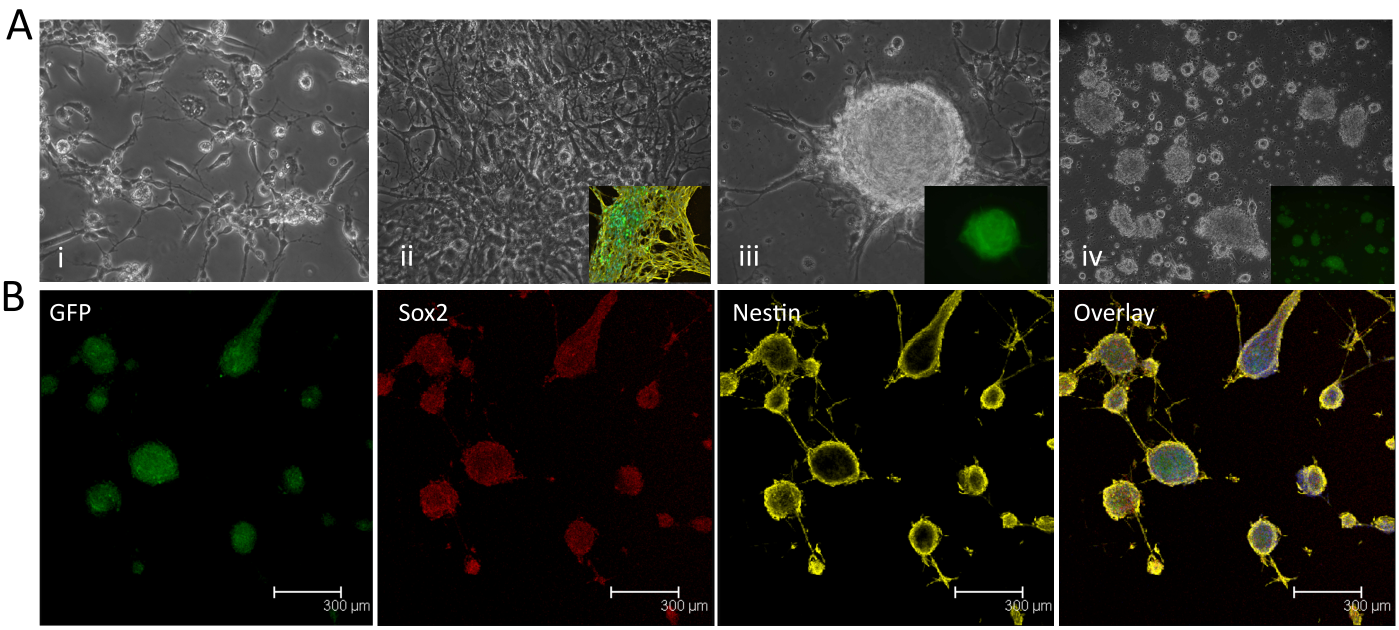


Fig. S12. A. White field images of SynI-Cre derived neurons maintained in Neurobasal media supplemented with B27 before (panel i) and after (ii) transduction with H-RasV12-shp53 lentivirus (inset in panel ii shows expression of MAP2 marker (yellow) and GFP (green/lenti-vector); magnification x20). Transduced neurons transferred to neural stem cell media supplemented with FGF-2 changed morphology and formed neurosphere like structures (panel iii magnification x40 and panel iv magnification x20; inset: green=GFP). B. Confocal microscopy analysis of neurospheres showing positive staining for Sox2 (red) and Nestin (yellow) markers.

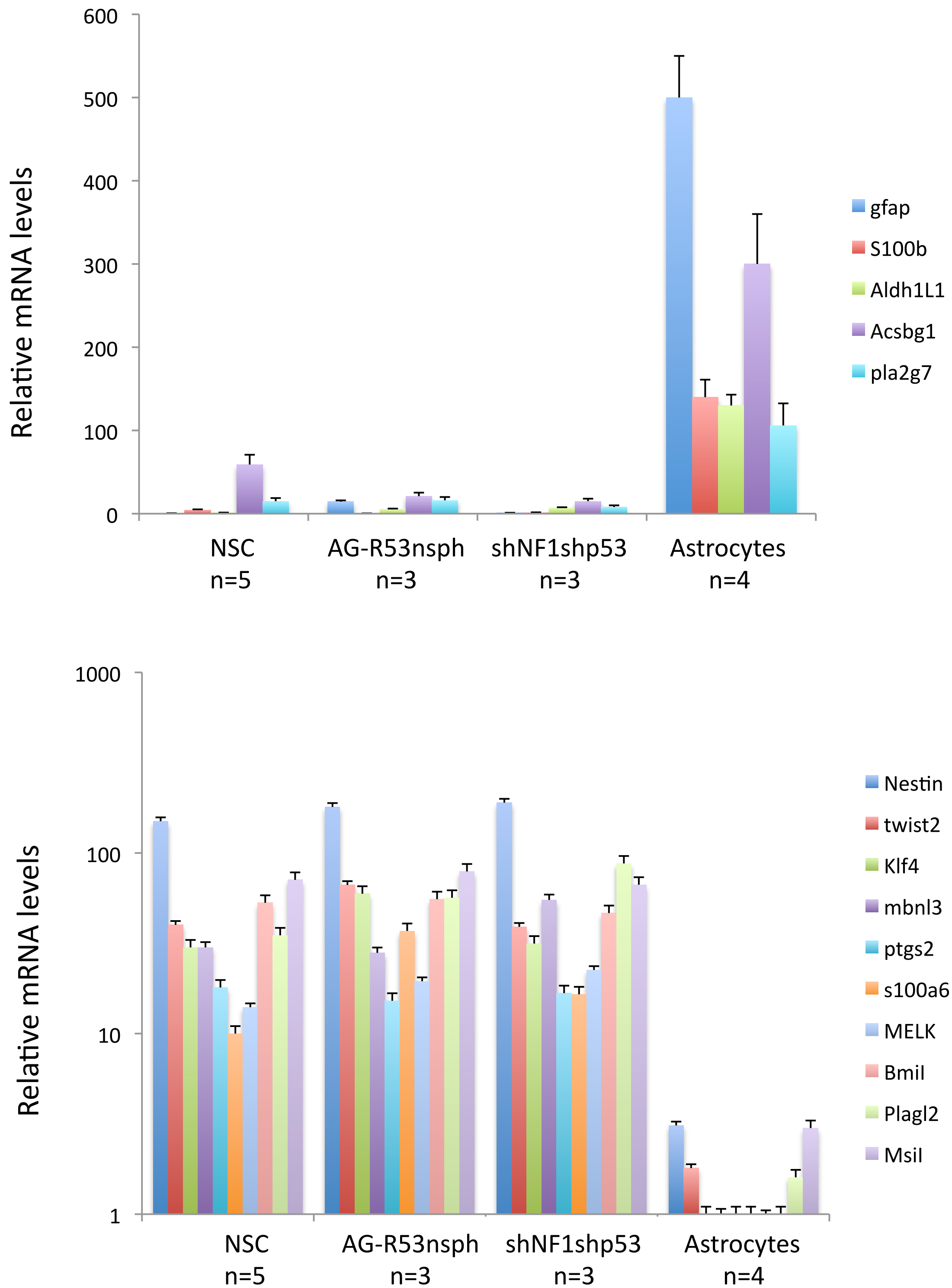
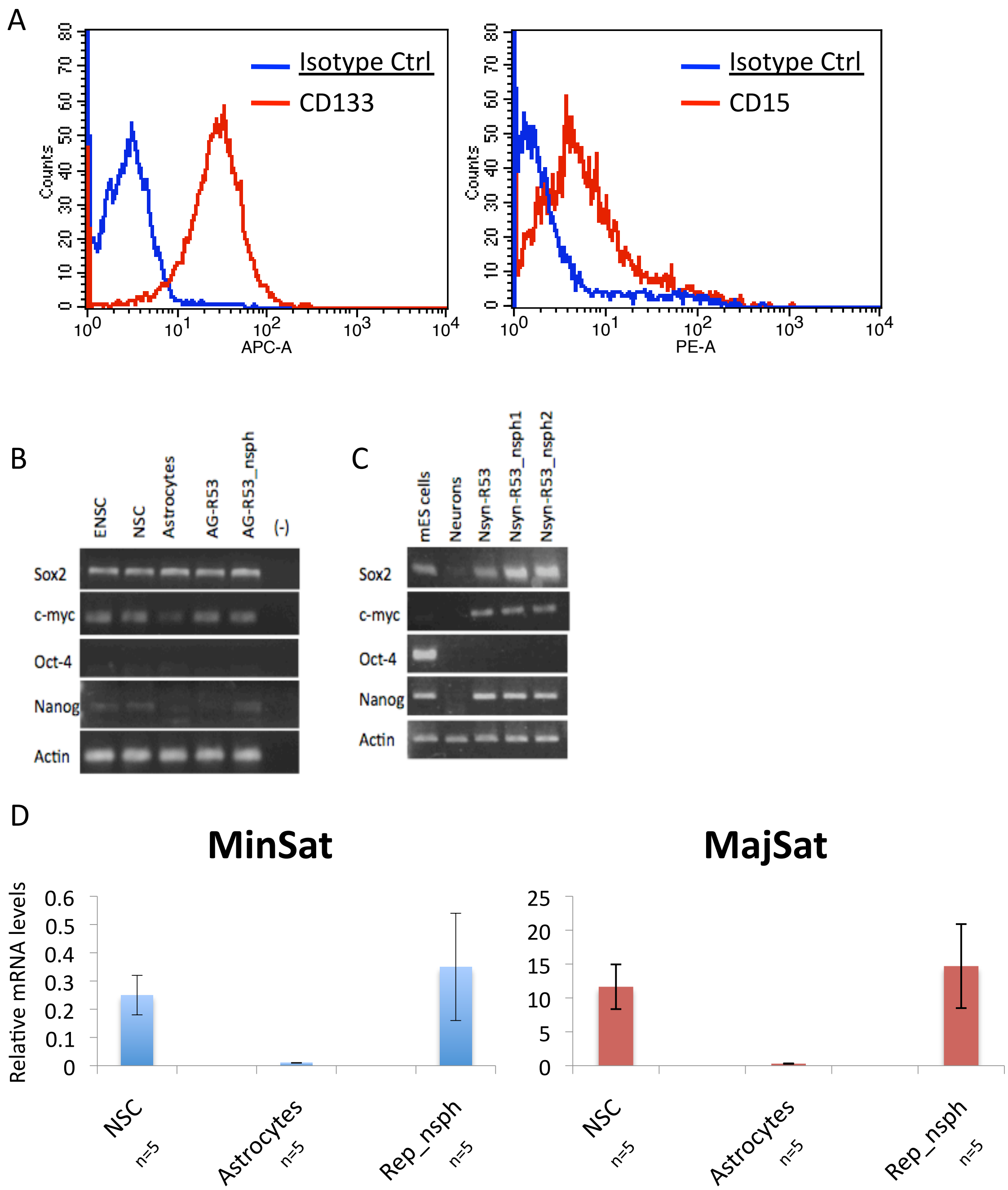
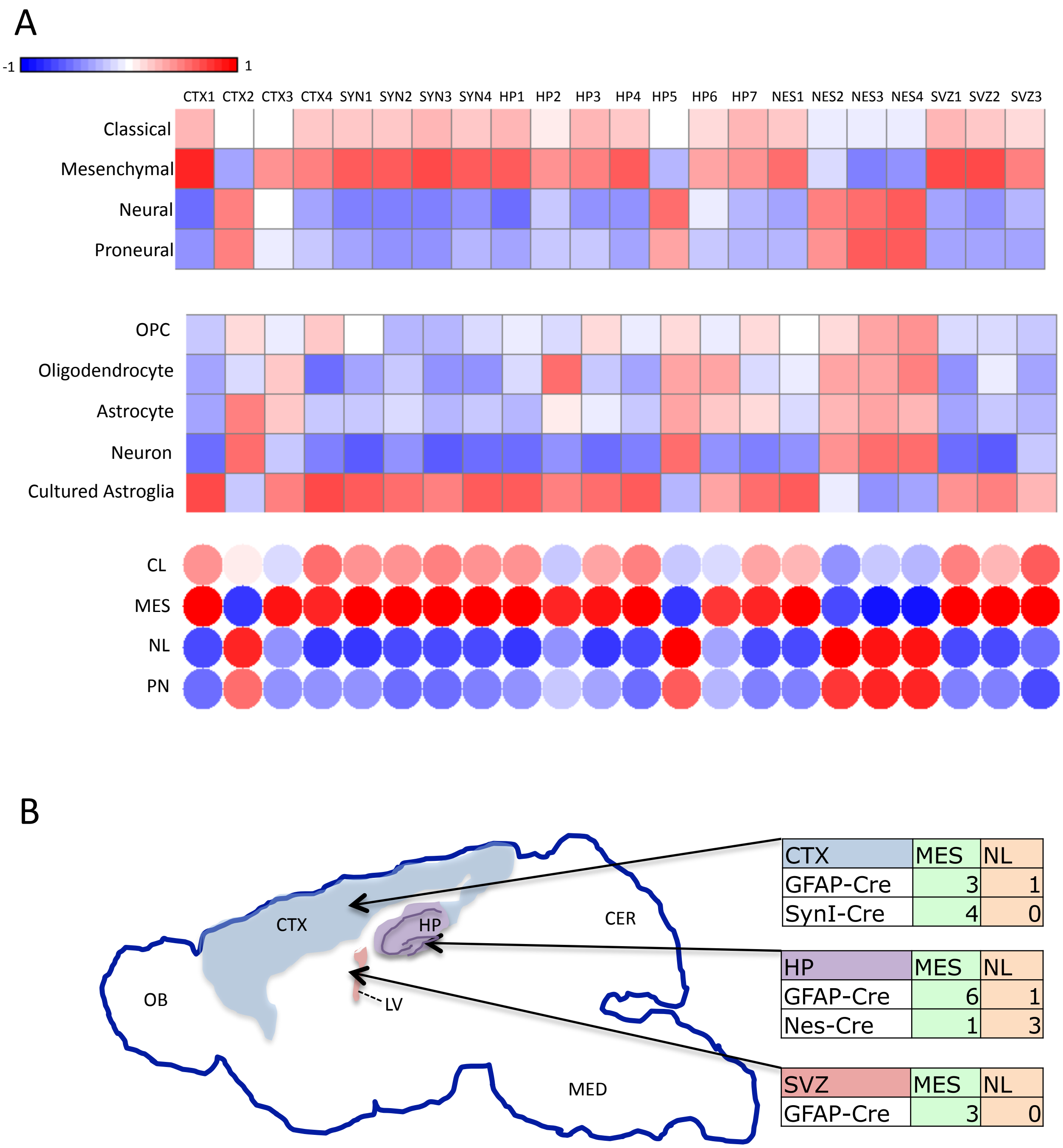


Fig. S13. Gene expression analysis by qRT-PCR for ten NSC- and five astrocyte- specific genes in adult NSC (n=5), GFAP-Cre derived astrocytes (n=4), three different GFP+ dedifferentiated NSCs using H-RasV12-shp53 (named AG-R53nsph) and three dedifferentiated NSC-like lines using shNF1-shp53 (named shNF1-shp53). Gene expression is normalized to the expression of cyclophilin.



Suppl. Fig 14. *Characterization of dedifferentiated GFP+ NSCs*. A) Flow cytometry analysis of GFP+ dedifferentiated neurospheres (AG-R53_nsph) using antibodies against CD133 (left panel, red line) and CD15 (right panel, red line), blue line is isotype matched controls in both panels. B) Embryonic Neural stem cells (ENSC), Neural stem cells (NSC) and AG-R53_nsph (transduced astrocytes with H-RasV12 switched to NSC media+FGF-2) were cultured in NSC media, whereas Astrocytes and AG-R53 (transduced astrocytes with H-RasV12-shp53) were cultured in DMEM+10%FCS. RT-PCR shows basal reprogramming factors expression. C) Same experiment as A but this time Neurons and NSyn-R53 were cultured in "neurons' media" and the rest of the indicated cell lines were cultured in NSC media. D) Quantitative RT-PCR showing that certain chromatin regions are exposed in NSC as well as in all the dedifferentiated neurospheres (Rep_nsph).


A
-1
1
CTX1 CTX2 CTX3 CTX4 SYN1 SYN2 SYN3 SYN4 HP1 HP2 HP3 HP4 HP5 HP6 HP7 NES1 NES2 NES3 NES4 SVZ1 SVZ2 SVZ3
Classical
Mesenchymal
Neural
Proneural
OPC
Oligodendrocyte
Astrocyte
Neuron
Cultured Astroglia
CL
MES
NL
PN
B
CTX
HP
CER
OB
LV
MED
CTX | MES | NL
GFAP-Cre | 3 | 1
SynI-Cre | 4 | 0
HP | MES | NL
GFAP-Cre | 6 | 1
Nes-Cre | 1 | 3
SVZ | MES | NL
GFAP-Cre | 3 | 0

C

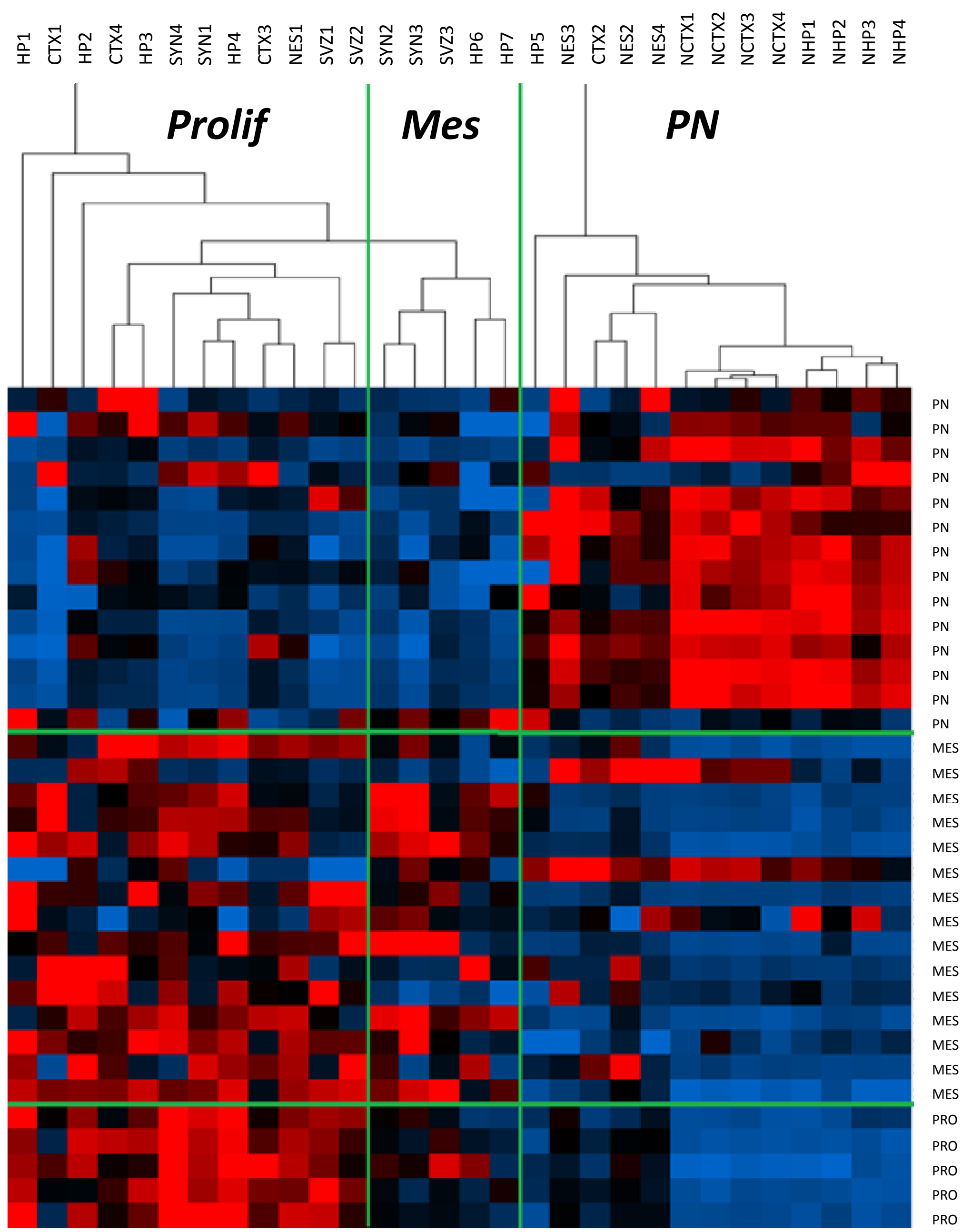


Fig S15. *Gene expression data identify two GBM subtypes in lentivirus-induced tumors*. A. Molecular signatures of GBM subtypes and murine brain cell types were projected onto harvested mouse tumors using a modified version of single sample GSEA. Enrichment scores (squares) for each signature are normalized for display purposes by the maximum possible score given a similarly sized gene list and gene set. Correlation coefficients (circles) were used for the combined mouse molecular signatures to the average signatures of human tumors found in Verhaak et al (*25*) to assign a final GBM subtype classification: Classical (CL), Mesenchymal (MES), Neural (NL), and Proneural (PN). In all cases a positive score, either enrichment or correlation coefficient, indicates a positive correlation whereas a negative score indicates the reverse. B. Summary diagram showing the site of injections for each transgenic strain and the resulting GBM molecular subtype for the analyzed tumors. C. Same line of comparison but using Phillips *et al* (*22*) molecular signatures. Abbreviations: olfactory bulb (OB), cortex (CTX), hippocampus (HP), lateral ventricle (LV), subventricular zone (SVZ), cerebellum (CER), medulla (MED), SynapsinI (SYNI), Nestin (NES).

Table S1. Quantification of transduced neurons in SynI-Cre mice

Number of GFP+/RFP- cells	NeuN+	GFAP+	
25	25	0	
		Ki67+	
20	20	0	
		Olig2+	
21	21	0	
		Nestin+	
26	26	0	
	Tuj1+	GFAP+	Nestin+
25	25	0	0

SynI-Cre mice (n=10) were injected in the cortex with H-RasV12-shp53 virus and 5 days after infection the mice were euthanized and the brain collected and fixed. Sections of the brain were first scanned for GFP+/RFP- cells and then co-labeled with the antibodies indicated in the table. Cells were picked randomly across the mice and different sections, and counted.

Table S2 - **Summary of stereotaxic injections with H-RasV12-shp53 lentivirus**

Location	Genotype	No. of mice	Latency	Tumors
HP	GFAP-Cre	50	6-10 weeks	50/50
CTX	GFAP-Cre	20	6-10 weeks	20/20
SVZ	GFAP-Cre	10	6-10 weeks	10/10
STR	GFAP-Cre	10	8-10 weeks	10/10
HP	Nestin-Cre	20	6-10 weeks	20/20
SVZ	Nestin-Cre	15	6-10 weeks	15/15
CTX	Nestin-Cre	15	NT*	1/15
STR	Nestin-Cre	5	NT	0/5
HP	Sox2-Cre	10	6-10 weeks	10/10
CTX	Sox2-Cre	5	NT	0/5
STR	Sox2-Cre	5	NT	0/5
SVZ	Sox2-Cre	10	6-10 weeks	10/10
CTX	Camk2a-Cre	6	9-12 months	6/6
CTX	SynI-Cre	20	6-10 weeks	20/20

HP= hippocampus, CTX=cortex, SVZ=sub-ventricular zone, STR=striatum, NT= No Tumor
* Only one mouse developed GBM with a latency of 10 months.

Table S3. List of Antibodies

Antibody	Manufacturer
GFAP	Neuromics
GFAP	Dako
Nestin	Neuromics
Nestin	Abcam
Nestin	BD Pharmingen
NG2	Millipore
Tuj1	Covance
NeuN	Millipore
Flag (M2)	Sigma Aldrich
CRE	Covance
S100b	Swant
MAP2	Sigma Aldrich
DCX	Santa Cruz
Sox2	Cell Signalling
NF1	Abcam
p53	Abcam
p-AKT	Cell Signalling
AKT	Cell Signalling
Tubulin	Santa Cruz
Ki67	Vector
O4	Gift from R. Gage
RIP	Gift from R. Gage
CD133	eBioscience
PE-conjugated anti-SSEA1	eBioscience
anti-b-galactosidase	Promega
anti-b-galactosidase	Cappel
anti-mouse IgG-HRP	Santa Cruz
anti-rabbit IgG- HRP	ECL
Cy3 anti-guinea pig	Jackson ImmunoResearch
Alexa 488 anti-rabbit IgG	Invitrogen
Alexa 568 anti-rabbit IgG	Invitrogen
Alexa 647 anti-rabbit F(ab')	Invitrogen
Alexa 488 anti-goat	Invitrogen
Alexa 568 anti-mouse IgG	Invitrogen
Alexa 488 anti-mouse IgG	Invitrogen
Alexa 647 anti-chicken IgG	Invitrogen
anti-chicken RRX	Jackson ImmunoResearch

Table S4. List of qRT-PCR primers

	5' primer	3' primer
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
twist2	CGCTACAGCAAGAAATCGAGC	GCTGAGCTTGTCAGAGGGG
MELK	TATGAAACGATTGGGACAGGTG	CCCTAGCGCATTCTTATCCATGA
klf4	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT
mbnl3	TTGATTCACTAAAGGGTCGGTG	CTGCGGCAGTCTTCTGTTGA
ptgs2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
Bmi-1	AAATCCCCACTTAATGTGTGTCC	GGCATCAATGAAGTACCCTCCA
Msi1	CCTCTCACGGCTTATGGGC	CTGTGGCAATCAAGGGACC
s100b	TGGTTGCCCTCATTGATGTCT	CCCATCCCCATCTTCGTCC
Aldh1L1	CAGGAGGTTTACTGCCAGCTA	CACGTTGAGTTCTGCACCCA
Acsbg1	ATGCCACGCGGTTCTGAAG	GAGCTGGTTTGCGAGTTGTCT
Pla2g7	CTTTTCACTGGCAAGACACATCT	CGACGGGGTACGATCCATTTC

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